

**United States Department of Agriculture  
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Screening for Zeranol by ELISA		
Revision: .01	Replaces: 00	Effective: 12/08/2005

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**A. INTRODUCTION**

1. Theory

Homogenized sample tissues are incubated with glucuronidase solution to enzymatically hydrolyze bound zeranol residues. Free zeranol is then extracted from the hydrolysate with diethyl ether, and the ether extract is taken to dryness. Residues are redissolved in chloroform, and the zeranol residues are partitioned into a NaOH solution. After acidification, zeranol in the aqueous extract is partitioned back into ethyl ether. The ether is evaporated and the residue redissolved in an aqueous buffer. An aliquot of this solution is then analyzed using a RIDASCREEN® Zeranol competitive enzyme immunoassay (EIA) ELISA test kit.

2. Applicability

This method is applicable for the qualitative analysis of zeranol in liver, at levels  $\geq 0.5$  ppb.

**B. EQUIPMENT**

Note: Equivalent apparatus and instrumentation may be substituted for the following items.

1. Apparatus

a. RIDASCREEN® Zeranol Test Kit -

Each kit contains sufficient materials for 96 measurements (including standard analyses). Each test kit contains:

- i. 1 x Microtiter plate (12 strips with 8 removable wells each) coated with antibodies directed against anti-zeranol antibodies.

Note: Keep unused wells together with the drying agent and well sealed in the foil bag. Store in refrigerator.

- ii. 6 x Zeranol standard solutions, 1.3 mL each, 0 ppt (zero standard), 31.25 ppt, 125 ppt, 500 ppt, 2000 ppt, 8000 ppt zeranol in aqueous solution, ready to use.

Note: A value of less than 0.6 absorbance units ( $A_{450\text{ nm}} < 0.6$ ) for the zero standard may indicate deterioration of reagents.

- iii. 1 x Conjugate (0.7 mL), peroxidase conjugated zeranol concentrate, red cap.

Note: The zeranol enzyme conjugate (bottle with red cap) is provided as a concentrate. Since the diluted enzyme conjugate has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the enzyme conjugate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1+10) in buffer

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(e.g. 200 µL conjugate concentrate + 2 mL buffer, sufficient for 4 microtiter strips).

- iv. 1 x Anti-zeranol-antibody (0.7 mL), concentrate, black cap.

The anti-zeranol antibody (bottle with black cap) is provided as a concentrate. Since the diluted antibody solution has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the antibody concentrate should be shaken carefully. For reconstitution, the antibody concentrate is diluted 1:11 (1+10) in buffer (e.g. 200 µL antibody concentrate + 2 mL buffer, sufficient for 4 microtiter strips).

- v. 1 x Substrate (7 mL solution), contains urea peroxide, green cap.

- vi. 1 x Chromogen (7 mL), contains tetramethylbenzidine, blue cap.

The colorless chromogen is light sensitive, therefore, avoid exposure to direct light. Any coloration of the chromogen solution is indicative of deterioration and the reagent should be discarded.

- vii. 1 x Stop solution (14 mL) contains 1 N sulfuric acid, yellow cap.

- viii. 1 x Buffer (50 mL), conjugate, antibody and sample dilution buffer.

Note: Store the kit in the refrigerator. Do not freeze. Bring all reagents to room temperature (20 - 25 °C) before use. Return all reagents to the refrigerator immediately after use.

- b. Incubator - 37 °C, Lab-line Instruments, Model #100.
- c. Nitrogen evaporator (N-Evap) - Model 112, Organomation Associates, Inc, 266 River Road West, Berlin, MA.
- d. Micropipettes - Adjustable 0 µL to 100 µL, (Gilson Pipetman P100) and 500 µL to 1000 µL, (Labnet VE-1000XR).
- e. Pipette tips - 1 - 200 µl, Fisher brand, Redi-Tip General Purpose, 21-197-8E, and 1000 µL Fisher brand, Redi-Tip, 21-197-8A.
- f. Balance - top loader, Mettler PC 440, Hightstown, New Jersey.
- g. Centrifuge - Damon IEC Centra GP8R, International Equipment Company, Needham Heights, MA.
- h. Adjustable Volumetric delivery devices - 1 - 5 mL, and 1 - 10 mL, Brinkman Dispensett 13-986-525D.
- i. Centrifuge tubes - 50 mL screw cap Number 8422, Pyrex.
- j. Borosilicate disposable culture tubes - Fisherbrand, Number 14-961-27.
- k. Explosion Proof ISOtemp combination refrigerator/freezer - Fisher Scientific, 13-986-525D.
- l. Mechanical shaker - flat bed, Eberbach Corporation, Ann Arbor, Michigan,

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- m. Vortexer - Thermolyne Maxi MixPlus, Model Number M63215, Barnstead International 2555 Keeper Blvd, Dubuque, Iowa.
- n. Ultra low temperature freezer - Capable of at least - 25 °C, Harris, SLT10LS90APP.

2. Instrumentation

Microtiter plate spectrophotometer - 450 nm. BioTek Instrument EL x 808, Highland Park, Winooski, VT.

**C. REAGENTS AND SOLUTIONS**

Note: Equivalent reagents and solutions may be substituted for the following items.

1. Reagents

- a. Methanol - J.T. Baker, 9093-03.
- b.  $\beta$ -glucuronidase - Sigma, G-7770.
- c. Diethyl ether - Aldrich 17, 926-4, Milwaukee, WI.
- d. Chloroform - Fisher Optima, C297-4.
- e. Deionized water - US Filter, Number PL5112.
- f. Sodium hydroxide (NaOH) pellets - J.T. Baker, 3728-05.
- g. Acetic acid (glacial) - J.T. Baker, 9507-000, Phillipsburg, N.J.

2. Solutions

- a. 1 M NaOH:  
Weigh 20 g NaOH and dissolve with distilled water. Bring to volume with distilled water in a 500 mL volumetric flask.
- b. 90% Acetic acid:  
Add 50 mL distilled water and 450 mL acetic acid to a 500 mL volumetric flask. Mix.

**D. STANDARDS**

1. Source

- a. The external standards used are supplied with the kit at levels of 0, 0.5, and 2.0 ng/mL.
- b. Zeranol ( $\alpha$ -zearalanol) - Sigma, Z 0292, St. Louis, MO.

Note: Equivalent standard and solutions may be substituted for any of the following.

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2. Preparation

- a. Zeranol stock solution (200 µg/mL):  
Weigh 20 mg of zeranol into a 100 mL volumetric flask. Dilute to volume with methanol.
- b. Zeranol working solution (0.2 ng/µL):  
Dispense 100 µL of zeranol stock solution into a 100 mL volumetric flask. Dilute to volume with methanol.
- c. Fortification solution (0.02 ng/µL):  
Dispense 10 mL of zeranol working solution into a 100 mL volumetric flask. Dilute to volume with methanol.

Note: Allow standards to equilibrate to room temperature before use.

3. Storage and Stability

- a. The stock solutions are stable for 1 year if kept in freezer
- b. The working standard solutions are stable for up to 6 months if kept in freezer.
- c. The kit standards have an expiration date set by the manufacturer and should be stored in the refrigerator with the kit.

**E. SAMPLE PREPARATION**

Homogenize liver samples and freeze prior to extraction if analysis is not begun on the same day as preparation.

**F. ANALYTICAL PROCEDURE**

1. Extraction Procedure

- a. Weigh 1 g of ground sample into a 50 mL screw cap centrifuge tube. Weigh 2 blank samples to be used as a blank and fortified control. For fortified control, add 25 µL of 0.02 ng/µL fortification solution for a concentration of 0.5 ppb.
- b. Add 2 mL of distilled water.
- c. Shake vigorously for 10 minutes.
- d. Add 8 µL of β-glucuronidase and incubate for 2 hours at 37 ±1 °C.
- e. Add 10 mL diethyl ether, cap, and shake vigorously to check for leaks.
- f. Shake for another 10 min and then centrifuge for approximately 10 min / ≅3,000 g / ≅15 °C.
- g. Freeze samples at - 25 °C for 60 min or at least - 60 °C for 30 min or freeze vial on dry ice / acetone. Decant the ether layer to a centrifuge tube.

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- h. Allow the aqueous phase to thaw, add 5 mL diethyl ether, shake vigorously and repeat extraction as in steps f-g.
- i. Evaporate pooled ether extracts to dryness (at  $60 \pm 3$  °C preferably under a gentle stream of nitrogen).  
Stopping point: Dried residues obtained from step F. 1. i can be stored capped and frozen for up to 1 month.
- j. Dissolve dried residue in 1 mL chloroform.
- k. Add 3 mL of 1 M sodium hydroxide and shake vigorously for 30 sec.
- l. Centrifuge: approximately 10 min /  $\cong 2,000$  g /  $\cong 15$  °C.
- m. Pipet the sodium hydroxide extract (upper aqueous layer) into a vial containing 250  $\mu$ L of 90% acetic acid.
- n. Add 5 mL diethyl ether, and shake vigorously for 30 sec.
- o. Centrifuge: approximately 10 min /  $\cong 2,000$  g /  $\cong 15$  °C.
- p. Freeze at - 25 °C for 60 min or at least -60 °C for 30 min or freeze vial on dry ice / acetone and decant the ether phase into a glass vial.
- q. Evaporate to dryness (at  $60 \pm 3$  °C preferably under a gentle stream of nitrogen).  
Stopping point: Dried residues obtained from step F. 1. q can be stored capped and frozen for up to 1 month.
- r. Dissolve the dried extract in 0.5 mL of sample dilution buffer.  
Note: A further dilution of the extract, up to 1:10, in sample dilution buffer may be made to decrease matrix effects.  
Stopping point: The ready to use extract obtained from step F. 1. r can be stored capped at 2 - 8 °C for up to 3 days.

2. Test Procedure

- a. Insert a sufficient number of wells into the microwell holder for all external standards and samples to be run in duplicate. Record standard and sample positions. The 0.5 ppb fortified control should be randomly placed into 6 wells throughout the plate.
- b. Add 50  $\mu$ L of each standard solution or prepared sample to separate duplicate wells. Add 50  $\mu$ L of diluted enzyme conjugate to each well.
- c. Add 50  $\mu$ L of the diluted anti-zeranol antibody solution to each well. Mix gently by rocking the plate manually and incubate 2 h at room temperature (20 - 25 °C).  
Note: Avoid direct sunlight during all incubations. Covering the microtiter plates is recommended.
- d. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete

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removal of liquid from the wells. Fill all wells with 250 µL of distilled water and pour out the liquid again. Repeat two more times.

- e. Add 50 µL of substrate and 50 µL of chromogen to each well. Mix gently by rocking the plate manually and incubate for 30 min at room temperature (20 - 25 °C) covered in the dark.
- f. Add 100 µL of the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 60 minutes after addition of stop solution.

3. Instrumental Settings

Set photometer at 450 nm.

**G. CALCULATIONS**

1. Evaluate sample results based on absorbance values for fortified control tissue. Zeranol concentration is inversely related to intensities of yellow color.
2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six 0.5 ppb control replicates. Use these to determine a decision level (DL) using the formula  $DL = \text{Mean} + 3 \times SD$ . Identify the sample as positive if its average absorbance is less than the decision level.

**H. SAFETY INFORMATION AND PRECAUTIONS**

1. Required Protective Equipment - Lab coat, safety glasses and protective gloves.
2. Hazards

Procedure Step	Hazard	Recommended Safe Procedures
Stop solution	Fumes are corrosive. Spattering may result in serious eye, skin, and respiratory damage.	Prepare solutions in a well-ventilated area such as a fume hood and dispense using repipettors wherever possible. Wear plastic gloves.

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3. Disposal Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Stop solution	See above	Neutralize and flush down the sink. Observe all Federal, state and local environmental laws.

**I. QUALITY ASSURANCE PLAN**

1. Performance Standard

- a. A plate must meet all of the following criteria:
  - i. The external standard curve has absorbances continuously increasing from the 2.0 ng/mL through each lower concentration standard through the EIA buffer.
  - ii. The absorbances increase from the 0.5 ppb to the 0 ppb fortified control tissue wells.
  - iii. No false positives for the negative control.
  - iv. The absorbance of the zero standard must be > 0.6 absorbance units or at the level specified in the kit.

2. Critical Control Points and Specifications

- a. Do not use RIDASCREEN® Zeranol kit past the expiration date on the kit label. Dilution or adulteration of these reagents may result in loss of sensitivity.
- b. Do not interchange individual reagents between kits of different lot numbers.
- c. Do not allow microwells to dry between working steps.
- d. Reproducibility in any EIA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the EIA test procedure.

3. Readiness To Perform (FSIS Training Plan)

- a. Familiarization
  - i. Phase I: Standards- Duplicate standard curves on each of 3 consecutive days, which will include the following:
    - (a) 0 ng/mL
    - (b) 0.5 ng/mL
    - (c) 2.0 ng/mL



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- ii. Phase II: Fortified samples - Triplicate analyses at 0, 0.5, and 1.0 ppb over a period of 3 different days using bovine liver.  
Note: Phase I and Phase II may be performed concurrently.
    - iii. Phase III: Check samples for analyst accreditation.
      - (a) 20 unknown beef liver samples. The sample fortifications including the number of blanks are to be blind to the analyst. 6 - 10 of the 20 samples should be blank and the rest fortified at 0.5 ppb.
      - (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM).
      - (c) Letter from QAM is required to commence official analysis.
  - b. Acceptability criteria.
    - i. No false negatives determined for controls at the 0.5 ppb level.
    - ii. Refer to section I.1 above.
4. Intralaboratory Check Samples
- a. System, minimum contents.  
Frequency: 1 weekly per analyst as samples analyzed.
  - b. Acceptability criteria.  
Same as 3. b. above.  
If unacceptable values are obtained, then:
    - i. Stop all official analyses by that analyst.
    - ii. Take corrective action.
5. Sample Acceptability and Stability
- a. Matrix: Beef liver
  - b. Sample receipt size: Approximately 200 g.
  - c. Sample receipt condition: Frozen
  - d. Sample storage:
    - i. Sample:  $\leq 6$  months if stored frozen.
    - ii. Extract:
      - (a) Dried residues obtained from step F. 1. h and F. 1. p can be stored frozen for up to 1 month.
      - (b) The ready to use extract obtained from step F. 1. q can be stored at 2 - 8 °C for up to 3 days.

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6. Sample Set

- a. Each sample set must contain:
  - i. Matrix Blank
  - ii. Fortified control
  - iii. Samples

7. Sensitivity

- a. Minimum proficiency level (MPL): 0.5 ppb.

**J. WORKSHEET**

The following worksheet is an example.

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[illegible]

Position Number	Lab Number	Form Number	Wt. of sample 1.00 ± 0.10 g	Result (+) or (-)
25				
26				
27				
28				
29				
30				
31				
32				
33				
34				
35				
36				
37				
38				
39				
40				
41				
42				
43				
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47				
48				

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**K. APPENDIX**

1. Cross-reactivity:

Zearalanone.....ca. 2 %  
β-Zearalanol.....ca. 4 %  
Zearalenone..... < 0.5 %  
α-Zearalenol.....ca. 21 %  
β-Zearalenol.....ca. 2.5 %  
17β-Estradiol..... < 0.02 %  
Clenbuterol..... < 0.02 %  
Testosterone ..... < 0.02 %

2. Reference

RIDASCREEN® Zeranol, 02-06-12.

Note: If package insert changes, use up to date instruction sheet from the test kit.

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**Approvals**

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Approvals are on file.